Enzyme and Microbial Technology 85 (2016) 90–97

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Synthesis of octyl-β-D-glucopyranoside catalyzed by Thai rosewood β-glucosidase-displaying *Pichia pastoris* in an aqueous/organic two-phase system

DongHeng Guo, YanShan Xu, YaJun Kang, ShuangYan Han*, SuiPing Zheng*

Guangdong Key Laboratory of Fermentation and Enzyme Engineering, School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China

ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 15 July 2015
Accepted 23 July 2015
Available online 26 July 2015

Keywords:
Thai rosewood β-glucosidase
Yeast whole-cell catalyst
Transglycosylation
Octyl-β-D-glucopyranoside
Scale synthesis

ABSTRACT

We explored the ability of a Thai rosewood β-glucosidase-displaying *P. pastoris* whole-cell biocatalyst (Pp-DCBGL) system to synthesize alkyl β-D-glucosides. The primary investigation centered on the synthesis of octyl-β-D-glucopyranoside (octyl-glu, OG). OG could be synthesized through reverse hydrolysis reaction with very low efficiency. Then, OG was synthesized between BG and octanol by a transglycosylation reaction. In a 2-ml reaction system, OG was synthesized with a conversion rate of 51.1% in 3 h when 5 mg/ml BG was utilized as the glucosyl donor under optimized conditions. And, even after being reused four times, the Pp-DCBGL was relatively stable. Additionally, a 500-ml-scale reaction system was conducted in a 2-L stirred reactor with a conversion rate of 47.5% in 1.5 h. Moreover, the conversion rate did not decrease after the whole-cell catalyst was reused two times. In conclusion, Pp-DCBGL has high reaction efficiency and operational stability, which is a powerful biocatalyst available for industrial synthesis.

1. Introduction

Alkyl β-D-glucosides are a group of completely biodegradable, moderate, stable, non-ionic and non-toxic low surface tension surfactants, which have foaming, wetting, detergent and emulsifying properties [1]. Alkyl β-D-glucosides have already been widely used in cleaning agents, pharmaceuticals, and food products [2], as well as in the field of protein science [3] and in cosmetics [4] and personal care products. Furthermore, alkyl glucosides with short and long hydrocarbon chains have different properties depending on the length of their chains [5,6]. From the perspectives of ecology and energy, they are the most promising new generation of surfactants.

Compared with the chemical synthesis of alkyl β-D-glycosides, the enzymatic synthetic method is very promising. This procedure is catalyzed by β-glucosidases (EC 3.2.1.21), is stereo- and region-specific, and is easily carried out under mild conditions, without requiring the protection and deprotection of the hydroxyl groups, use of toxic catalysts and solvents, or isolation of useless byproducts [7]. Moreover, the separation of the targeted product from a complex mixture of anomers remains another major bottleneck of the chemical methods [8]. For β-glucosidases, the synthesis can be carried out by thermodynamic direct condensation between a monosaccharide and an alcohol (reverse hydrolysis) [9–11] or by kinetically controlled transglycosylation between a reactive glycosyl donor and an alcohol [12,13]. The chemical reaction equations of reverse hydrolysis and transglycosylation are listed below:

$$\text{reversehydrolysis}: \text{Gly-OH} + R^1\text{OH} \rightarrow \text{Gly-OR}^1 + H_2O \qquad (1)$$

where Gly-OH represents glucose, as the glycosyl donor, R^1OH represents fatty alcohol, and Gly-OR^1 represents alkyl β-D-glucosides.

$$\text{transglycosylation}: \text{Gly-OR}_2 + R_1\text{OH} \rightarrow \text{Gly-OR}_1 + R_2\text{OH} \qquad (2)$$

where Gly-OR$_2$ represents short-chain alkyl β-D-glucosides, as the glycosyl donor, R^1OH represents fatty alcohol, and Gly-OR^1 represents long-chain alkyl β-D-glucosides.

The transglycosylation reaction usually generates higher yields than reverse hydrolysis, perhaps due to its overshoot of the equilibrium for the conversion from reactants to products [14]. However, in a single-phase organic system with co-solvents [15], the efficiency of transglycosylation has been limited by low enzyme stability and poor solubility of the substrates. Furthermore, the reaction efficiency is also lowered by high mass transfer resistance. Such problems can be avoided or reduced in a water/alcohol two-

---

* Corresponding authors at: School of Bioscience and Bioengineering, South China University of Technology, Guangzhou City, 510006, China.
E-mail addresses: syhan@scut.edu.cn (S. Han), spzheng@scut.edu.cn (S. Zheng).

http://dx.doi.org/10.1016/j.enzmictec.2015.07.006
0141-0229/© 2015 Published by Elsevier Inc.

phase reaction system. Moreover, in this system, the enzymes can make very good contact across the interface with the substrates that are dissolved in both phases [16].

As a cost-effective bioconversion process, an enzyme-displaying cell provides a new, more reasonable approach for lowering the costs of industrial synthesis. The enzymes are firmly displayed on the cell surface due to their connection with cell-wall-anchored proteins [17]. The whole-cell biocatalyst, which can be considered a type of self-immobilization, can be produced through high-density fermentation and without any protein purification and immobilization.

Thai rosewood β-glucosidase (also called dalcochinase, DCBGL), which was purified from Thai rosewood seeds, has shown high levels of β-glucosidase activity, compared with β-glucosidases isolated from other plant seeds [18]. Svasti [19,20] has demonstrated that DCBGL can catalyze the synthesis of oligosaccharides and alkyl-β-ᴅ-glucosides via transglycosylation with water-immiscible alcohols. However, until now, the majority of DCBGL used for catalysis research was derived directly from the seeds of Thai Rosewood, a procedure that was economically inefficient. No report has detailed the use of a whole-cell biocatalyst system with Thai rosewood β-glucosidase-displaying *P. pastoris* (Pp-DCBGL) for the synthesis of alkyl glucosides.

In the present study, we constructed a recombinant strain displaying DCBGL on the surface of *P. pastoris* cells. We explore the alkyl glucoside synthetic ability of a DCBGL-displaying *P. pastoris* whole-cell biocatalyst (Pp-DCBGL) system. We mainly focused on the synthesis of octyl-β-ᴅ-glucopyranoside (OG), as catalyzed by Pp-DCBGL, and utilized an alcohol-water two-phase reaction system. Several reaction parameters were optimized, including the pH, water content, temperature, and concentrations of the whole-cell biocatalyst and substrate. Subsequently, scaled-up reactions were performed in a stirred reactor under optimized conditions.

2. Materials and methods

2.1. Strains and materials

Octyl-β-ᴅ-glucopyranoside (octyl-glu,OG) and 4-nitrophenyl-β-ᴅ-glucopyranoside (*p*NPG) were purchased from Sigma–Aldrich (St. Louis, USA) and used as standards. Other chemicals were commercially obtained and were of analytical grade.

Escherichia coli Top10 was used as the host strain for plasmid storage and amplification. *P. pastoris* GS115 was used for cell surface display. The recombinant *Candida antarctica* lipase B (CALB) surface-displaying plasmid pKCALB-GCW21 was previously constructed by Jin et al. [21] in our laboratory. The recombinant plasmid pKDCBGL-GCW21 and DCBGL-displaying *P. pastoris* strain were constructed for this study.

E. coli was cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/ml kanamycin. Yeast strains were grown on either an MD plate (2% glucose, 1.34% YNB, 2% agar) or in BMGY/BMMY media (1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 400 μg/L biotin, 1% glycerol or 2% methanol). The *P. pastoris* GS115 strain was cultured under the following conditions: 30 °C, 250 rpm.

2.2. Construction of the pKDCBGL-GCW21 expression plasmid and recombinant yeast strain

Gcw21p (NCBI accession No. XM_002491407) has been used as an anchor for the display of DCBGL on the *P. pastoris* cell surface. The recombinant expression plasmid pKDCBGL-GCW21 that was used for cell-surface display of Thai rosewood β-glucosidase was constructed as follows: DNA sequences encoding mature DCBGL with an HA peptide tag at the N-terminus were amplified by PCR. The upstream and downstream primers were 5′-CG*GAATTC*ATTTACCCATACGATGTTCCAGATTACGCTGATTTCGCCAAAGAAG-3′ and 5′-CG*ACGCGT*CTAAAATCC CTCAATACCTCTC3′, respectively, while plasmid pUC57/*dcbgl* was used as the template. The PCR products were digested with *Eco*R I/*Mlu* I and then introduced into the corresponding sites of the surface-displaying plasmid pKCALB-GCW21 as a replacement for the CALB gene.

The recombinant plasmid pKDCBGL-GCW21 was linearized and subsequently integrated into the host strain *P. pastoris* GS115. The transformant was selected by incubation at 30 °C for 72 h on MD plates. The isolated transformant was then precultured in BMGY medium at 30 °C. After 24 h, the culture was centrifuged at 6000 rpm for 5 min, and the cells were then resuspended in BMMY medium containing 2% (v/v) methanol while the initial OD_{600} was controlled at 1. To maintain the expression of the fusion proteins, methanol was added to the culture every 24 h to maintain the 2% (v/v) concentration. *P. pastoris* GS115 transformed with pPIC9K (GS115/pPIC9K) was used as a negative control.

2.3. Immunofluorescence analysis of recombinant yeast strain GS115/pKDCBGL-GCW21

As noted previously, DNA sequences encoding mature DCBGL have an HA peptide tag at the N-terminus. As the fusion protein was displayed on the yeast cell surface, the HA tag was also displayed on the cell surface. The HA tag specifically binds to the antibody that is conjugated to the fluorescent substance. By fluorescence microscopy and flow cytometry, expression of the fusion protein on the cell surface can be determined through cell surface fluorescence.

2.4. Preparation of the DCBGL-displaying P. pastoris whole-cell biocatalyst

The DCBGL-displaying recombinant *P. pastoris* cells were collected after 168 h of methanol induction. Subsequently, the cells were resuspended in a 0.2 M sodium acetate buffer (pH 5.0) with 0.2 M trehalose added as a protectant for freeze-drying. The suspension was lyophilized in a Christ Alpha 2-4 Freeze Dryer (Christ, Osterode, Germany) for 24 h. This procedure yielded the whole-cell biocatalyst that was used for subsequent experiments.

The hydrolysis activity was measured with 1 mM *p*NPG in 0.2 M sodium acetate (pH 5.0) in a 500-μL reaction at 50 °C for 10 min. The reaction was stopped by adding 500 μL of 2 M sodium carbonate, and the *p*-nitrophenol that was released was measured by its absorbance at 405 nm. One unit of hydrolysis activity was defined as the amount of biocatalyst required to release 1 μmol of *p*-nitrophenol from *p*NPG per min at 50 °C. Using this method, the hydrolysis activity of the yeast whole-cell biocatalyst (Pp-DCBGL) was 244 U/(g dry cell).

The synthetic ability of the whole-cell biocatalyst was analyzed using *n*-octanol and BG as substrates. To this end, 2.5 mg of the whole-cell biocatalyst was mixed with 1.85 ml *n*-octanol and 0.15 ml 0.2 M sodium acetate (pH 3.0) in a 25-ml stoppered glass Erlenmeyer flask. The mixture was then incubated on a rotary shaker at 50 °C and 200 rpm for 10 min. Next, 10 mg BG was added to the flask, and the mixture was incubated on a rotary shaker at 50 °C and 200 rpm for 45 min. One unit of synthesis activity corresponded to the amount of biocatalyst that produced 1 μmol of OG per min from *n*-octanol and BG at 50 °C. A control reaction was performed using the same procedure and the whole-cell but without DC-BGL. Using this method, the synthetic ability of Pp-DCBGL was 79.32 U/(g dry cell).